[Frontiers in Bioscience E4, 1263-1275, January 1, 2012]

Role of the RNA-binding protein Hfq in *Serratia plymuthica*

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1. ABSTRACT

The RNA-binding protein Hfq has been well studied as a global post-transcriptional regulator which controls diverse cellular processes in bacteria. However, the function in the genus of *Serratia* has remained unexplored. Here we show that beyond mutation in Hfq resulting in their growth defects, Hfq has global effects on a variety of biocontrol-related phenotypes in the endophytic strain G3 of *Serratia plymuthica*, including antifungal activity, production of exoenzymes, as well as motility and biofilm formation. Especially for the first time, Hfq is observed to control biosynthesis of auxin indole-3-acetic acid (IAA) and antibiotic pyrrolnitrin (PRN), which are key determinants responsible for plant growth promotion and suppression of phytopathogens, respectively by G3. Additionally, Hfq is also required for the production of RpoS, a major stress sigma factor in G3. In contrast to *E. coli*, translation of *hfq* in G3 is positively autoregulated. Further investigation of the detailed mechanisms for regulation of IAA and pyrrolnitrin production by Hfq and its role in the regulatory networks of G3 will help to optimize the beneficial plant-microbe interactions.

2. INTRODUCTION

Hfq is a small, highly abundant RNA-binding protein that is mostly conserved among the bacterial kingdom, and belongs to the large family of Sm and Sm-like proteins in all three domains of life. Sm proteins are essential components of the small nuclear ribonucleoproteins (snRNPs) that form spliceosomes in eukaryotes (1). Hfq (HF-I) was initially discovered as a host factor required for phage Qbeta RNA replication in *Escherichia coli* about 40 years ago and forms a homohexameric structure that binds preferentially to A/U-rich RNAs (2). Hfq is now known to function as a pleiotropic post-transcriptional regulator that modulates the stability and translation of mRNAs by facilitating small RNA-mRNA interactions in bacteria (3). Beyond being an RNA chaperone, Hfq modulates the decay of some mRNAs, by binding to their poly(A) tails, stimulating poly(A) adenylation by poly(A) polymerase I (PAP I) and protecting this message from polynucleotide phosphorylase (PNP), RNase II and RNase E enzymes involved in mRNA degradation (4). The innate importance of Hfq in cellular function became clear when an *hfq* null mutant was

constructed in *E. coli* and showed pleiotropic effects, including decreased growth rates, increased sensitivity to ultraviolet light, mutagens and oxidants and increased cell length, as well as sigma E-mediated envelope stress response and the sigma32 (RpoH)-mediated cytoplasmic stress response (5, 6). Hfq has been shown to be required for the virulence and fitness of an increasing number of bacterial pathogens, for example, exopolysaccharide (EPS) synthesis in *Burkholderia cepacia* (2). Transcriptomic analysis revealed that Hfq controls, directly or indirectly, the expression of about 20% of all *Salmonella* genes for many biological processes including host cell invasion, motility, central metabolism, lipopolysaccharide (LPS) biosynthesis, two-component regulatory systems (TCS), and fatty acid metabolism (7, 8). The Hfq-dependent alterations of the transcriptome profile and effects on quorum sensing (QS) were also observed in *Pseudomonas aeruginosa* (9). Despite the recent works have showed that RNA chaperon Hfq plays a key role in the establishment of the symbiosis between free-living *Sinorhizobium meliloti* and its host alfalfa, as well as control of central carbon metabolism (10, 11), the role of the homologous *hfq* gene in plant beneficial bacteria and its contribution to biocontrol are less understood.

Plant-associated *Serratia plymuthica* belongs to the group of gamma–proteobacterial species of Gram-negative bacteria as a biocontrol agent (BCA) of mainly fungal pathogens, as well as entomopathogens and weeds. Multiple modes of action and their synergistic effects have been recognized for plant growth promotion and biocontrol (12, 13). Previous studies have shown that multiple global regulators including GacS/GacA homologues, GrrA/GrrS, the stationary-phase sigma factor RpoS, and *N*-acylhomoserine lactone (AHL)-mediated QS system are involved in control of biocontrol activities in *S. plymuthica* strains IC1270 and HRO-C48 (14-17). However, compared to transcription regulation, the post-transcriptional regulation has remained less explored. In the present study, our major aim was to investigate the role of the RNA-binding protein Hfq in expression of biocontrol-related phenotypes in an endophytic strain of *S. plymuthica* which will help to provide a new strategy for enhancement of biocontrol activity and plant growth promoting ability. Our results revealed that Hfq is involved in global regulation of various biocontrol traits in G3. To our best knowledge, the production of plant auxin indole-3-acetic acid (IAA) and antibiotic pyrrolnitrin (PRN) which are key determinants for plant growth promotion or suppression of phytopathogens was observed under the control of Hfq by G3 for the first time, as well as Hfq is positively autoregulated, in contrast to the autorepression of Hfq in *Escherichia* coli (18).

3. MATERIALS AND METHODS

3.1. Microorganism strains, plasmids and growth conditions

The bacterial and fungal strains, plasmids and primers used in this study are listed in Table 1. The endophytic strain *Serratia plymuthica* G3 was isolated from the stems of wheat in Shandong, China. Strain G3 with resistance to rifampin and its derivatives were grown in LB medium with appropriate antibiotics at 28°C and for *Escherichia coli* at 37°C with shaking at 180 rpm. Growth rates of G3 and its derivatives were monitored every three hours by measuring OD value at 600 nm. The fungal isolate *Cryphonectria parasitica* was from the authors' laboratory collection and was routinely cultured on potato dextrose agar (PDA) at 25°C.

3.2. DNA preparation and manipulations

Standard methods were used for plasmid and genomic DNA isolation, restriction enzyme digestion, agarose gel electrophoresis, ligation, and transformation (19).

3.3. Cloning and sequencing of the *hfq* gene from *S. plymuthica* strain G3

The gene encoding *S. plymuthica* Hfq was amplified from strain G3 using genomic DNA as a template by polymerase chain reaction (PCR). Pair of primers *hfq*-F and *hfq*-R in Table 1 for cloning the *hfq* locus was designed to the conserved regions of the corresponding genes related to *Serratia* in GenBank using the ClustalW multiple sequence alignment program. The following program was used for thermal cycling: 94°C for 4 min, then 30 cycles at 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min, and a final extension at 72°C for 10 min. After purification, PCR products were cloned into pMD19-T (Takara, Dalian, China) to give p19T-*hfq*.

3.4. Phylogenetic analysis

A phylogenetic tree was constructed using the neighbour-joining method of MEGA 4 (27). The predicted Hfq amino acid sequence from the G3 isolate was analyzed together with those from 25 species of other bacteria. These Hfq amino acid sequences were obtained from GenBank. The tree topology was tested by bootstrap analysis of 1000 samplings.

3.5. Mutant construction and complementation

To investigate the specific functions, the *hfq* insertion mutant was constructed by homologous recombination with the *sacB*-based strategy. Briefly, a 931-bp fragment bearing the gentamicin resistance gene *aac1*, encoding gentamicin acetyltransferase 3-1, was cut by *Sma*I from p34S-Gm and inserted into the *hfq* gene sequence of p19T-*hfq* where the site for insertion of the gentamicin cassette was *Nae*I, 231-bp away from the start coden ATG of the *hfq* gene. The interrupted gene was excised at the *Xba*I and *Sal*I sites and recloned into the same sites of the suicide vector pDM4. The resulting pDM4-*hfq*::Gm was introduced into the *E. coli* S17-1 strain by electroporation, then used as a donor to transfer the constructs into strain G3 by mating. The double-crossover progeny were selected on LB plates supplemented with 10% (wt/vol) sucrose, gentamicin (25 μg/ml), and rifampin (40 μg/ml), and sensitive to chloramphenical (25 μg/ml). The gene replacement was further verified by PCR cloning and sequencing using the genomic DNA of the candidate mutants as templates. All enzymes are from MBI Fermentas, Lithuania.

Table 1. Strains, plasmids and primers used in this study

Strain or plasmid	Relevant characteristics or sequence[1]	Reference or source
Serratia plymuthica		
G3 WT	Rif^r, isolated from stems of wheat in Taian, China	13
hfq- (*hfq*::Gm)	Rif^r, Gm^r, an *hfq* insertion mutant of strain G3	This study
hfq-/pUCP26-*hfq*	Rif^r, Gm^r, Tc^r, the complementary strain of the *hfq* mutant	This study
hfq-/pUCP26	Rif^r, Gm^r, Tc^r, the *hfq* mutant containing pUCP26 as control	This study
Escherichia coli		
DH5alpha	F− *recA1 endA1 hsdR17 deoR thi-1 supE44 gyrA96 relA1* D(*lacZYA*±*argF*) *U169 k*− [*u*80d*lacZ*DM15]	19
S17-1	*thi pro hsdR recA*; chromosomal RP4; Tra$^+$; Sm/Sp^r	20
Plasmids		
pMD19-T	Ap^r, cloning and sequencing vector	Takara
p19T-*hfq*	pMD19-T carrying the full *hfq* gene, Ap^r	This study
p34S-Gm	Source of Gm^r cassette	21
pDM-4	Cm^r, oriT$^+$ *sacB* suicide vector	22
pDM4-*hfq*::Gm	pDM4 carrying hfq::Gm, Gm^r,Cm^r	This study
pEXT20	Ap^r, the *tac* promoter	23
pUCP26	Tc^r, broad host range vector	24
p26-*hfq*	pUCP26 carrying the *hfq* gene under the control of P*tac*	This study
pBluelux	Ap^r, a promoter-less *luxCDABE* cassette in pBluescript II	25
pP_{prn}::*lux*	pUCP26 with a *prnA* promoter fusion to *luxCDABE*, Tc^r	This study
pME6015	pVS1-p15A shuttle vector for translational *lacZ* fusions, Tc^r	26
pME6015-*prnA*	pME6015 with a *prnA'-'lacZ* translational fusion, Tc^r	This study
pME6015-*rpoS*	pME6015 with a *rpoS'-'lacZ* translational fusion, Tc^r	This study
pME6015-*hfq*	pME6015 with a *hfq'-'lacZ* translational fusion, Tc^r	This study
Primers		
hfq-F	GCGCCAAATGACCTGGTT	This study
hfq-R	CACCGGCTTCATAACGGT	This study
Hfq-*hisF*	AGGATCCATGGCTAAGGGGCAATCT (*Bam*HI)	This study
Hfq-*hisR*	GAAGCTTTTAATGATGATGATGATGGTGTTCAGCGTCATCGCTTT (*Hind*III and 6-His-tag)	This study
A1	CGTAAGTAACGAATGAATC	This study
A2	CAGGCTAGACTCTCGTCT	This study
B1	CCGAATTCCGAATGTTGTTGAGTAT (*Eco*RI)	This study
B2	AAGGATCCCATCCAGCCCGCAGT (*Bam*HI)	This study
C1	GAATTCACTGCAACGGTTGATTCT (*Eco*RI)	This study
C2	GTCGACCTCGTTAACTTTCAGCGT (*Sal*I)	This study
D1	GAATTCGATCCATCAACGAATCG (*Eco*RI)	This study
D2	CTGCAGAAACCGGAACACGTTC (*Pst*I)	This study

[1]restriction enzyme recognition sites and histidine-tag are underlined

To complement the *hfq*- mutant and purify the Hfq protein in future for further investigation of the Hfq-RNA interactions, the *hfq* gene was cloned under the control of the P*tac* promoter and fused to a C-terminal histidine-tag. Briefly, the 341-bp of *hfq* gene with 6-His at the 3′-end was amplified using the primers Hfq-*hisF* and Hfq-*hisR* in Table 1 from G3 genome DNA, followed by cloned into pMD19-T. After digested with *Bam*HI and *Hind*III, the resulting fragment was subcloned into the corresponding sites of pEXT20 to enable the *hfq* gene under the control of the P*tac* promoter. Then the P*tac*-*hfq* fragment was excised with *Ssp*I and ligated into the *Sma*I site of the broad-host-range vector pUCP26. The complementation plasmid p26-*hfq* and the vector pUCP26 were introduced into the *hfq*- mutant, respectively.

3.6. Phenotypic analysis

3.6.1. Biocontrol-related phenotypes

Assays for detection of exoenzymes, motility and biofilm formation, as well as antifungal activity against *Cryphonectria parasitica in vitro* were performed as previously described (28-31).

3.6.2. Quantification of IAA production

Overnight cultures of *S. plymuthica* G3 and its derivatives in LB broth with appropriate antibiotics were diluted (OD_{600} 0.02) into 2 ml of LB with 200 μg/ml of tryptophan. After further incubation for 42 h, Salkowski's method was used to determine IAA production (32-33). A standard curve generated from known concentrations of IAA was used to calculate the concentration of IAA.

3.6.3. Thin layer chromatography (TLC) detection of PRN production and bioassay

For PRN extraction, 100 μl of fresh bacterial overnight cultures (adjusted to OD_{600} 1.0) were grown on LB agar medium with 2% glycerol in triplicate for 5 days at 28°C according to Costa et al. 2009 with minor modifications (34). Medium were cut into small pieces and transferred to 250 ml flasks containing 40 ml of ethyl acetate with shaking for 90 min at 37°C and allowed to stand for 5 min at room temperature. The solvent phase supernatant was collected, transferred to a new tube and allowed to dry. Dried bacterial extracts were resuspended in 30 μl of methanol. Each 10 μl of extract suspensions from G3 and the *hfq*- mutant were applied onto silica gel 60 F254 TLC plates (Merck KGaA, Darmstadt, Germany). Separation was performed with hexane-ethyl acetate (2:1 v/v) as the solvent system with pure PRN (Sigma-Aldrich, St Louis, USA) as positive control. The corresponding PRN spots were detected by spraying with 2% Ehrlich's reagent

(2% p-dimethylaminobenzaldehyde in 5N HCl). After drying, observed under UV_{254nm} and took a photo. For bioassay of antifungal activity against *C. parasitica*, each 4 µl of extract suspensions were applied in wells on PDA plates, and the equal amount of methanol was used as a negative control.

3.7. Construction and analysis of *lux*-based and *lacZ*-based fusions

To construct a *lux* transcriptional fusion to *prnA* promoter, a 503-bp fragment containing the *prnA* gene promoter region $P_{prnA,}$ was amplified using the primer pair A_1 and A_2 in Table 1 and genomic DNA of G3 as a template. The PCR fragment was digested with *Xba*I and *Sal*I, and ligated into the *Sma*I site of pBluelux after Klenow blunting. The positive clones were selected according to bioluminescence, followed by excised with *Pst*I and ligated into the same site of the broad-host-range vector pUCP26, the resulting pP_{prn}::*lux* was introduced into the wild type G3 and the *hfq*- mutant, respectively. A translational *prnA*′-′*lacZ* fusion was constructed by amplifying a 708-bp PCR product with primers B_1 and B_2 in Table 1. This fragment was digested with *Eco*RI and *Bam*HI and cloned into the corresponding sites of pME6015. In the resulting plasmid pME6015-*prnA*, the promoter region of the *prnA*, and the sequence encoding the first 19 amino acids of *prnA* are fused to the *lacZ* reporter, then was mobilized into strains G3 and the *hfq*- mutant, respectively by electroporation for further analysis of the influence of Hfq on expression of *prnA* gene.

Similarly a translational *rpoS*′-′*lacZ* or *hfq*′-′*lacZ* fusion was also constructed by PCR amplifying with the primer pairs C_1 and C_2 or D1 and D2 in Table 1 as described above in order to investigate the impact of Hfq on RpoS and its autotranslation. The resulting 630-bp PCR fragment containing the promoter region of the *rpoS* gene and the sequences encoding the first 10 amino acids, as well as the 496-bp PCR fragment containing the promoter region of the *hfq* gene and the sequences encoding the first 22 amino acids are fused to the *lacZ* reporter.

Bioluminescence was determined as a function of cell density with a combined luminometer/spectrophotometer (Bio-Tek, Massachusetts, USA) according to Atkinson et al. 2008 (29). Beta-galactosidase assays were performed using the substrate o-nitrophenyl-beta-D-galactoside (ONPG, Sigma-Aldrich, Inc.) as described by Miller (35). Each sample was assayed at least in triplicate. Data are mean values of three independent samples +/- SD.

3.8. Nucleotide sequence accession number

The GenBank accession number for the gene *hfq* from *S. plymuthica* strain G3 is EU812513**.**

4. RESULTS

4.1. Cloning of the *S. plymuthica* G3 *hfq* gene

The homologue of the RNA chaperon Hfq gene was cloned from strain G3 by PCR with conserved primers as described in the Material and Methods. The 649-bp PCR product was sequenced. The resulting sequence included the open reading frame (ORF) of the 309-bp-long *hfq* gene of *S. plymuthica* G3 (EU812513). It is predicted to encode a protein of 102 amino acids (ACF28536) with 100% and 84% identity to Hfq proteins from the complete genome sequencing *Serratia odorifera* 4Rx13 and the well studied *E. coli* strain K-12, respectively. The downstream of *S. plymuthica hfq* gene is the *hflX*-like gene coding for a putative GTP-binding protein, and they are conserved in the proteobacteria (11). The upstream of *hfq* is the gene coding for tRNA isopentenyltransferase. A multiple amino acid sequence alignment revealed that Hfq from strain G3 contains the conserved Sm1 motif, a characteristic of Sm and LSm (Sm-like) proteins that function in RNA processing events in achaea and eukaryotes, and Sm2 motif likely forming the RNA-binding pocket (1,11), as well as inter-hexamer interaction sites (data not shown). Further phylogenetic analysis with MEGA 4 showed that Hfq from *S. plymuthica* G3 is closely related to the other two *Serratia* species *S.odorifera* 4Rx13 and *S. proteamaculans* 568, and belongs to the beta/gamma-proteobacteria clade (Figure 1) with the conserved residues corresponding to the Hfq sequence positions 30, 43 and 50 from G3 are isoleucine, valine and valine as signature. Consistent with previous proposal to show that the Hfq proteins in bacteria shared a common ancestor with archaeal/eukaryotic Sm and LSm proteins, no evidence for lateral transfer of Hfq (1).

4.2. Construction of a *S. plymuthica hfq* mutant and complementation

An *hfq*- mutant was generated in the wild type strain G3 by disruption of *hfq* with the mobilizable suiside vector pDM4-*hfq*::Gm-mediated double crossing over. PCR amplification and sequencing verified the resulting *hfq*- mutant with the Gm cassette inserted after 231-nt of the *hfq* coding sequence. For complementation we used the wild-type *hfq* gene fused at its 3′-end to a His_6 tag and expressed under the control of the P*tac*. The resulting plasmid p26-*hfq* expressing the *hfq* gene from the *tac* promoter greatly complemented various phenotypes. However, it is worth noting that the *hfq* complementation in G3 was incomplete as reported previously in *E. coli* (36). We hypothesized that this defect in *hfq* complementation might be lack of positive autocontrol of *hfq* under the control of P*tac* instead of its native promoter as described below in 4.8, and still to be further verified.

4.3. Loss of Hfq results in growth defects of *S. plymuthica* G3

Hfq-dependent growth changes were first investigated by comparing the growth rate of wild type G3 and the mutant grown in LB medium. Overnight bacterial cultures were inoculated into glass tubes in 2 ml of LB (final concentration of OD_{600} 0.08) with shaking and incubated at 30C for 12 h. The cell densities were determined at 600 nm at interval of 3 h. The results showed that the mutant exhibited a severely reduced growth rate which was complemented through expression the *hfq* gene under the control of P*tac* promoter in the broad-host-range vector pUCP26 (Figure 2). In addition the colony forming unit (CFU) by plating for G3 and the *hfq*- mutant after incubation for 24 h with shaking is (2.7 +/- 0.1) $\times 10^{10}$ and

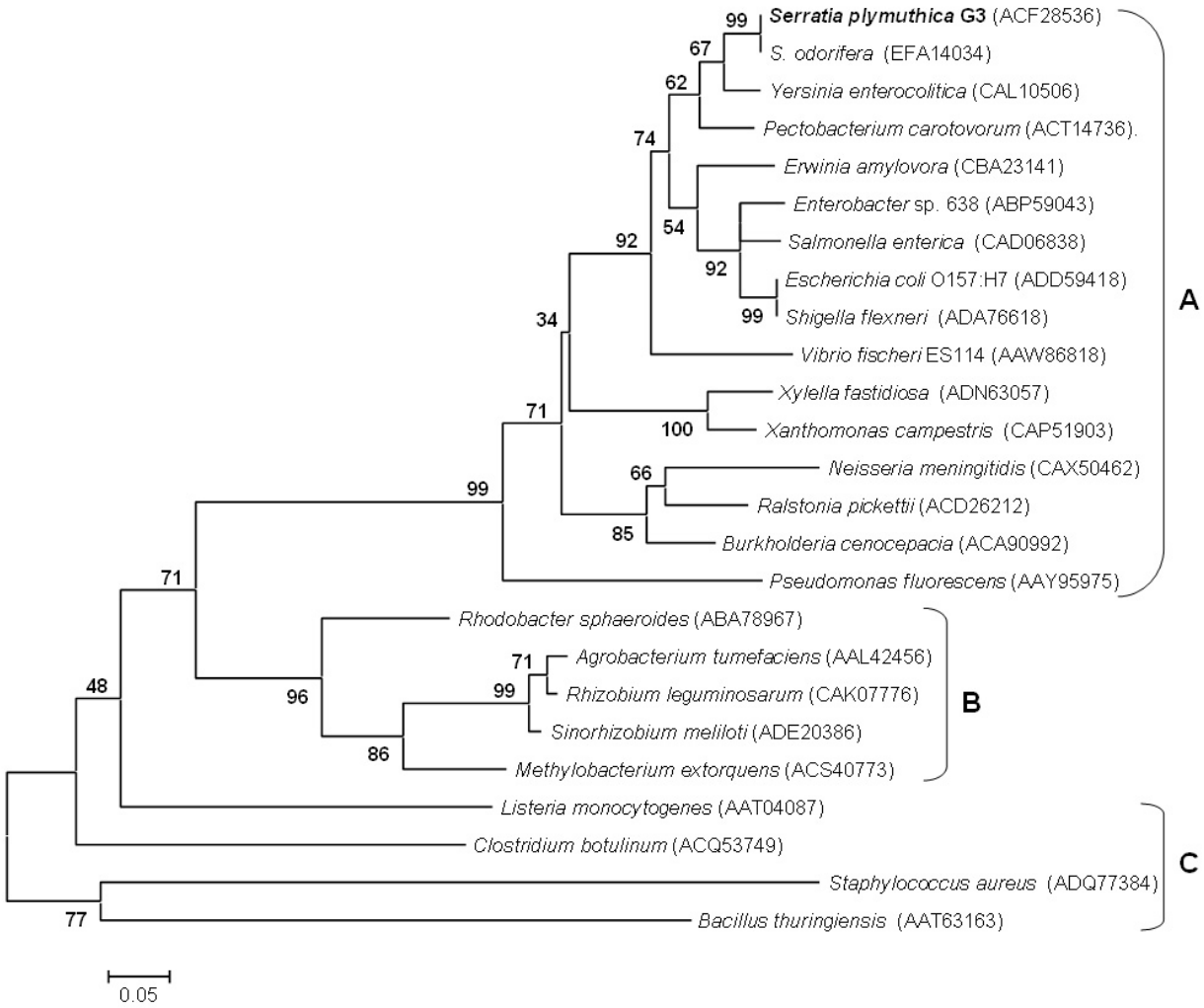

Figure 1. Neighbour-joining phylogenetic analysis of Hfq protein sequences using MEGA 4. The phylogenetic tree was constructed using Hfq protein sequences corresponding to the endophytic strain G3 and other representative species from a number of bacteria by a BLAST search NCBI database, followed by multiple sequence alignment by ClustalW. The significance of each branch is bootstrap value calculated for 1000 subsets. Scale bar indicates the mean number of substitutions per site. Members in group A belong to beta/gamma-proteobacteria; Members in group B belong to alpha-proteobacteria; Members in group C belong to Gram-positive bacteria.

(6.3 +/- 1.5) $\times 10^8$, respectively. These findings are consistent with previous discoveries that mutation in Hfq results in growth defect in a number of Gram negative bacteria (2, 5).

4.4 Hfq is required for expression of variety of biocontrol-related phenotypes

Although Hfq is a ubiquitous RNA-binding protein in bacteria and has been well studied, its function in biocontrol bacteria is still less known. Comparative analysis of biocontrol-related phenotypes between the wild type and the *hfq*- mutant were performed to unravel the role of Hfq in plant beneficial microorganism. The results revealed that Hfq as a pleiotropic regulator is involved in global regulation of biocontrol-related traits. As summarized in Table 2, Hfq is required for the production of chitinase and protease, swimming motility and biofilm formation, as well as antifungal activity against *Cryphonectria paracitica*. In addition, the swarming motility is also Hfq-dependent (Figure 3).

4.5. Hfq positively regulates accumulation of IAA by G3

Strain G3 with great potential of stimulating root development in *Arabidopsis* (Zhang H and Liu X. unpublished data) was detected to produce IAA (31). To test the impact of Hfq on auxin production by G3, the wild type G3 and the *hfq*- mutant were determine accumulation of IAA in the bacterial culture suspensions incubated at 28℃ for 42 h after entrance into stationary phase. As Figure 4 showed that the *hfq*- mutant significantly ($P<0.01$) decreased the level of IAA production compared with the wild type G3, although the complemented strain partially restored the IAA accumulation.

Table 2. Phenotypic characterization of strain G3 and its derivatives[1]

Phenotype	G3-WT	*hfq*::Gm mutant	*hfq*::Gm/p26-*hfq*	*hfq*::Gm/pUCP26
Antifungal activity (mm)	6.00 +/- 0.1 (A)	0 (C)	4.10 +/- 0.10 (B)	0 (C)
Protease activity (cm)	1.31 +/- 0.04 (A)	0 (C)	1.12 +/- 0.05 (B)	0 (C)
Chitinase activity (cm)	1.52 +/- 0.06 (A)	0 (B)	1.53 +/- 0.03 (A)	0 (B)
Swimming motility (cm)	2.06 +/- 0.15 (A)	0.81 +/- 0.04 (C)	1.94 +/- 0.02 (B)	0.80 +/- 0.05(C)
Biofilm formation (OD_{570})	0.39 +/- 0.01 (A)	0.15 +/- 0.02 (C)	0.25 +/- 0.01 (B)	0.14 +/- 0.02(C)

[1]Different letters indicate significant differences at P<0.01.

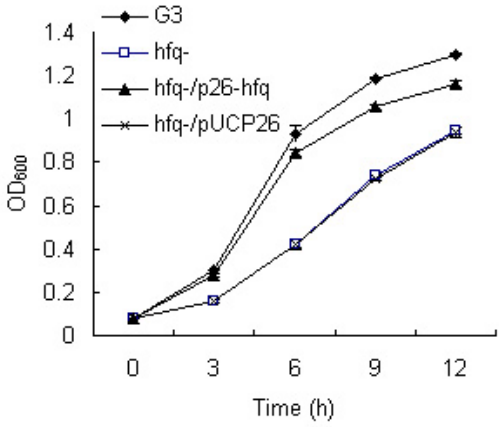

Figure 2. The growth curve of strain G3 and its derivatives. G3: Wild type, *hfq*-: the *hfq* minus mutant, *hfq*-/p26-*hfq*: the complemented strain and *hfq*-/pUCP26: the mutant carrying pUCP26 as a negative control.

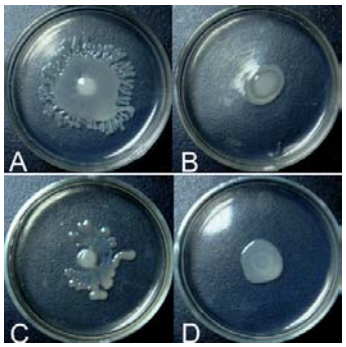

Figure 3. Effect of Hfq on swarming motility in G3. A: Wild type G3, B: the *hfq*- mutant, C: the complemented strain with pUCP26-*hfq*, D: the *hfq*- mutant with pUCP26 as control.

4.6. Hfq controls PRN production at the both transcriptional and translational levels

TLC analysis of PRN extracts demonstrated that mutation in *hfq* almost abolished the PRN production as compared with the wild type (Fig.5-A). Additionally the bioassay on the PRN extracts against *C. parasitica* also verified that the *hfq*- mutant lost the antifungal activity (Figure 5-B).

For further investigation of the role of Hfq in regulation of the *prnA* expression at the both transcriptional and post-transcriptional levels, we constructed a *lux*-based *prn*A promoter fusion and a translational *prnA*'-'*lacZ* fusion. Firstly, the resulting plasmid pUCP26/P_{prn}::*lux* was transferred to the wild-type G3 and the *hfq*- mutant. The gene expressional level based on *lux* fusions was determined by recording bioluminescence during 24 h in liquid culture in LB at 28C. The results revealed that mutation in *hfq* resulted in significantly reduced expression level of *prnA* promoter (Figure 6-A). Furthermore, the expression of *prnA* at the translational level mediated by plasmid pME6015-*prnA* in the wild-type G3 and the *hfq*- mutant was followed by measuring beta-galactosidase activity along the growth curve. In the wild-type strain the expression of the translational *prnA*'-'*lacZ* fusion was increased in a manner of cell density-dependent, but the *hfq*- mutant maintained very low level of beta-galactosidase activity, consistent with the observation by TLC detection of PRN extracts (Figure 5). These findings implied that Hfq controls the production of PRN at the both transcriptional and translational levels.

4.7. Hfq is required for expression of the *rpoS* gene

Previous studies (37-39) has well documented that Hfq is required for the efficient translation of the *rpoS* gene encoding sigma 38 , a alternative sigma factor

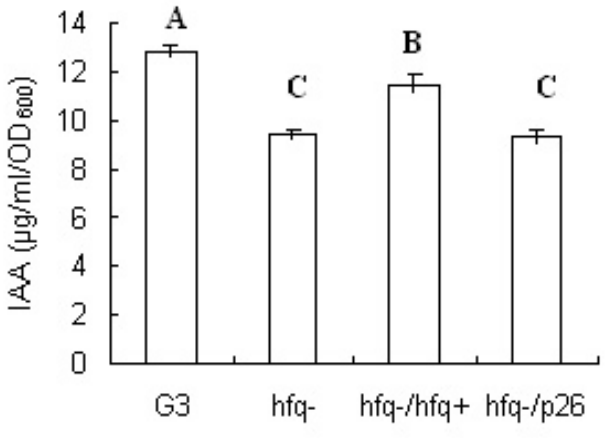

Figure 4. Hfq positively controls IAA production. G3: Wild type, hfq-: the *hfq* mutant, hfq-/hfq+: the complemented strain with pUCP26-*hfq*, hfq-/p26: the *hfq*- mutant with pUCP26 as control. Different letters indicate significant differences at P<0.01.

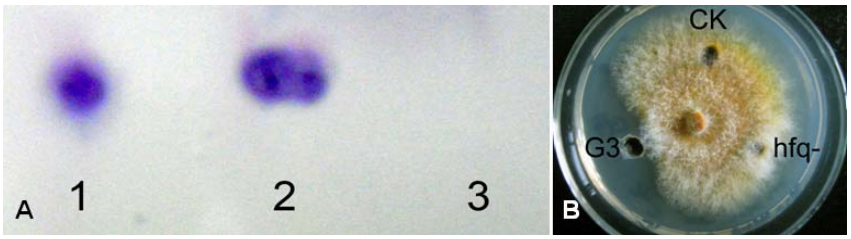

Figure 5. Detection of PRN production by TLC (A). 1: 1 μl of PRN standard (2 μg/ml), 2: 10 μl of PRN extracts from G3, 3: 10 μl of PRN extracts from hfq- mutant. Bioassay of 2 μl of PRN extracts from G3 and the *hfq*- mutant against *Cryphonectria parasitica* (B). CK: 2 μl of solvent methanol as control.

expressed under a variety of stress conditions and in stationary phase in bacteria. To verify the role of Hfq in translation of *rpoS* gene of *S. plymuthica*, we constructed a translational *rpoS'-'lacZ* fusion. Beta-galactosidase activity assays showed that the expression level of the translational *rpoS'-'lacZ* fusion in the backgroud of *hfq*- mutant was greatly decreased as compared with the wild type strain carrying the pME6015-*rpoS* (Figure 7). This finding indicated that Hfq also controls translation of the *rpoS* gene in biocontrol strain of *S. plymuthica*. Further multiple sequences alignment of the upstream region of the *rpoS* mRNA leader from gamma-proteobacteria revealed that the upstream Hfq binding elements $(ARN)_4$ and A_6 are conserved among these bacteria, where R is a purine nucleotide and N is any nucleotide (Figure 8), implying that Hfq in G3 might share similar regulatory mechanisms on the RpoS to that in *E. coli* where Hfq is require for RpoS production through binding the *rpoS* mRNA leader to recruit sRNAs to release the translation repress (37-39).

4.8. Hfq is positively autoregulated by itself

The translational autorepression of the *E. coli* Hfq has been reported (18). To determinate whether Hfq from strain G3 is also autocontrol, the *hfq'-'lacZ* translational fusion was constructed, wherein the first 496 nt of *hfq* mRNA containing the promoter region and the sequences encoding the first 22 amino acids of Hfq protein were fused to the eighth coden of the *lacZ* reporter gene. Assay of beta-galactyosidase activity demonstrated that Hfq is positively autoregulated via comparative analysis of expression of the *hfq'-'lacZ* translational fusion in the background of the wild type and the *hfq*- mutant after incubation for 12 and 24 hours. As Figure 9 illustrated that the beta-galactosidase activity decreased significantly in the *hfq*- mutant when compared with the wild type.

5. DISCUSSION

There has been increasing evidence for extensive post-transcriptional control of gene expression mediated by RNA-binding proteins in bacteria. A prominent example is Hfq that is found in many bacteria and plays a critical role in post-transcriptional gene regulation by facilitating pairing between small RNAs and mRNAs; Hfq also plays a key role in bacterial RNA decay by binding tightly to polyadenylate [poly(A)] tracts (2, 37, 39). Hfq has known to be involved in stress resistance and pathogenicity in a variety of bacterial pathogens (8; 40). Mutants lacking Hfq are often sensitive to host defense mechanisms and highly attenuated in animal models. Recent works demonstrated that Hfq in plant-associated *S. meliloti* is also required for the establishment of symbiosis with its legume host alfalfa and nitrogen fixation. Proteomic analysis showed that most differential expression of proteins in the free-living *S. meliloti hfq* mutant are involved in cell metabolism or stress resistance (10, 11, 41, 42). However, its role in plant

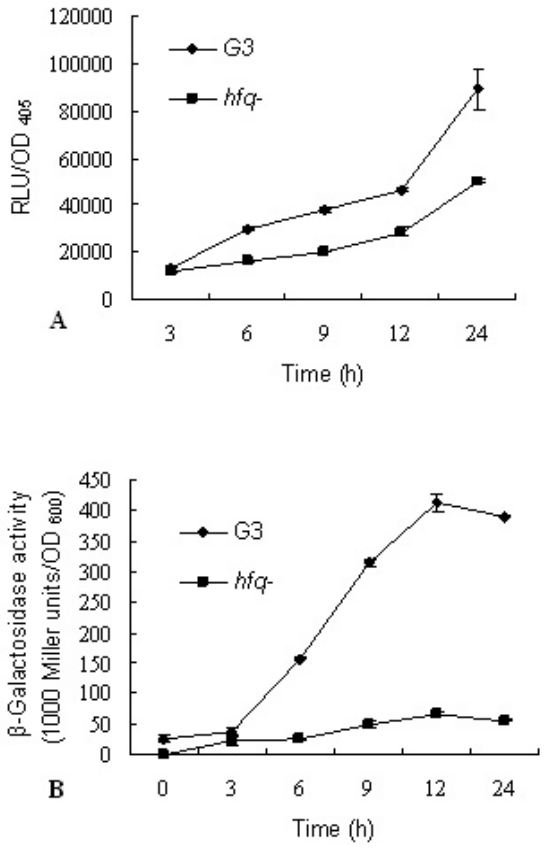

Figure 6. Hfq controls pyrrolnitrin production at the both transcriptional and translational levels. Based on a transcriptional *prnA* promoter::*lux* fusion (A) and a translational *prnA'-'lacZ*-fusion (B).

beneficial biocontrol bacteria is only poorly understood. Here we show that the Hfq from endophytic *S. plymuthica* G3 also functions as a pleiotropic regulator to control bacterial growth and biocontrol-related phenotypes. Hfq is required for extracellular proteolytic and chitinolyitic activities, and antifungal activity against *C. parasitica in vitro*. Loss of Hfq is shown to abolish biofilm formation and swarming motility, as well as decrease swimming motility. Furthermore, we provide the first experimental evidence that the production of plant auxin IAA or antibiotic PRN which is responsible for plant growth promotion or suppression of phytopathogens, respectively is also Hfq-dependent, although the regulatory molecular basis by Hfq remains to be further investigated. In contrast to *E. coli*, Hfq is positively autoregulated by *S. plymuthica* G3.

Phenotypic characterization and global transcriptome/proteome analyses of the *hfq* mutants have found their growth defects due to the extensive role of *hfq* in the regulation of nutrient uptake and central metabolism in numerous Gram-negative bacteria, such as *Brucella abortus*, *E. coli*, *P. aeruginosa*, *Salmonella typhimurium*, *Vibrio vholerae, Yersinia pestis*, and the symbiotic bacterium *S. meliloti*, although disruption of *hfq* in the Gram-positive bacteria, *Listeria monocytogenes* and *Staphylococcus aureus* causes no obvious growth defect (2,11). As predicted, our results reconfirmed that the insertion mutant of *hfq* in *S. plymuthica* significantly reduced growth rate compared with the wild type and was greatly complemented through *in trans* expression of P*tac-hfq*, indicating loss of Hfq resulting in growth defect is generic in Gram-negative bacteria. By contrast, Hfq in the biocontrol strain 2P24 of *Pseudomonas fluorescent* very recently has been reported to regulate production of the antibiotic 2,4-diacetylphloroglucinol (2,4-DAPG) and AHL signals at the transcriptional level, unexpectedly no obvious influence on bacterial growth was observed (43).

In biocontrol strains of S. *plymuthica*, compared to transcription regulation, the mechanisms affecting the regulation of translation remain less understood. Chitinases and proteases are produced by a wide range of S. *plymuthica* strains, and chitinases produced by *S. plymuthica* played an important role in the antifungal activity (44, 45). Research on the regulatory mechanisms demonstrated that the TCS *grrA* and *grrS* mutants of IC1270 were deficient in production of protease and endochitinase (14). Furthermore, expression of chitinase and protease is positively regulated by QS in strains HRO-C48 and RVH1 (16, 46). Our results demonstrated that the post-transcriptional regulator Hfq is also essential for both chitinolytic and proteolytic activities in *S. plymuthica* G3.

Swarming motility and biofilm formation is a type of multicellular behavior in bacteria, which are notable for their high resistance to environmental stresses, such as desiccation and antimicrobials, as well as to host defences (47). Therefore, biofilm formation is important for the persistence and survival of bacteria. In *Serratia marcescens* MG1 and *S. plymuthica* G3, biofilm formation has been demonstrated to be positive regulated by AHL-mediated QS (31, 48). However, the impact of Hfq on biofilm formation seems dependent on organism, For example, Hfq promotes biofilm formation in UPEC, but Δ*hfq* mutant in *Moraxella catarrhalis* seems to overproduce biofilm (2). Our data showed that both motility and biofilm formation are Hfq-dependent in biocontrol strain G3, implying its potential of Hfq in rhizospheric competence and colonization of host plant.

It has been well established that phytohormone IAA regulates plant growth and development, and also plant's response to biotic and abiotic stresses. The interest in microbial synthesis of auxin is also increasing due to the contribution to their host's hormone pool (49). A new intriguing role of auxin was suggested recently that microbially produced IAA can act as a reciprocal signaling molecule in microbe-plant interactions. Although reports on IAA biosynthesis in bacteria are numerous, regulation of IAA biosynthesis is only documented in a few bacteria. For example, autoinduction of IAA is observed in *Azospirillum brasilense* Sp245. Genetic factors including the altenative sigma factors RpoS in *Pseudomonas putida* GR12-2, and RpoN in Sp245, as well as the transcriptional regulator TyrR involved in transport and metabolism of aromatic amino acid in *Enterobacter cloacae* UW5 have been

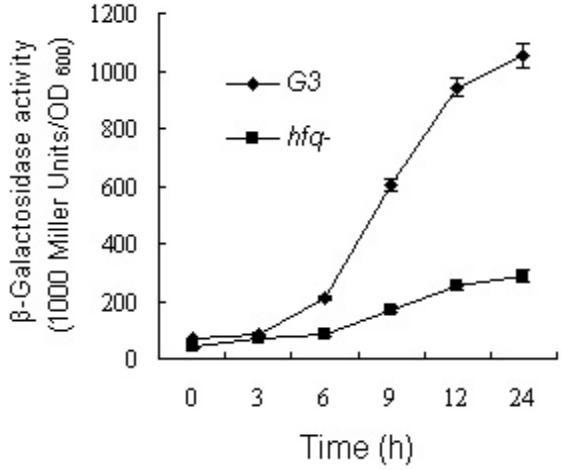

Figure 7. Hfq positively regulates RpoS production. The experiments based on comparative analysis of beta-galactosidase activities when expression of a *rpoS'-'lacZ* translational fusion in the wild type G3 and the *hfq*- mutant.

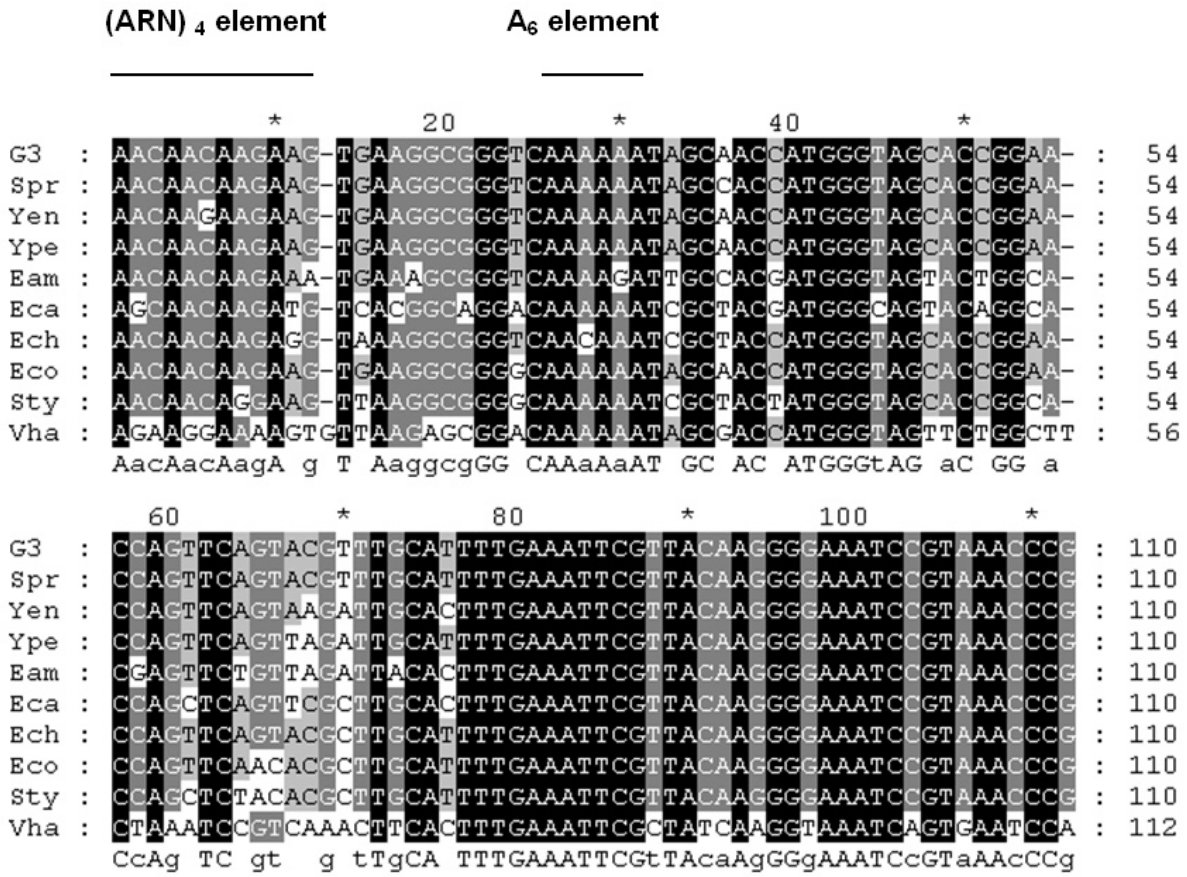

Figure 8. Multiple sequence alignment of the upstream region of the *rpoS* leader. An alignment of the *rpoS* leader from *S. plymuthica* and other Gram-negative gamma-proteobacteria was conducted using ClustalW. The Hfq-binding sequence motifs $(ARN)_4$ and A_6 elements are indicated, where R is a purine nucleotide and N is any nucleotide. G3: *Serratia plymuthica* G3, Spr: *Serratia proteamaculans* 568; Yen: *Yersinia enterocolitica* 8081; Ype: *Yersinia pestis* Z176003; Eam: *Erwinia amylovora* CFBP1430, Eca: *Erwinia carotovora* SCC3193, Ech: *Erwinia chrysanthemi* 3937, Eco: *Escherichia coli* UM146, Sty: *Salmonella typhi* Ty2, Vhe: *Vibrio harveyi*.

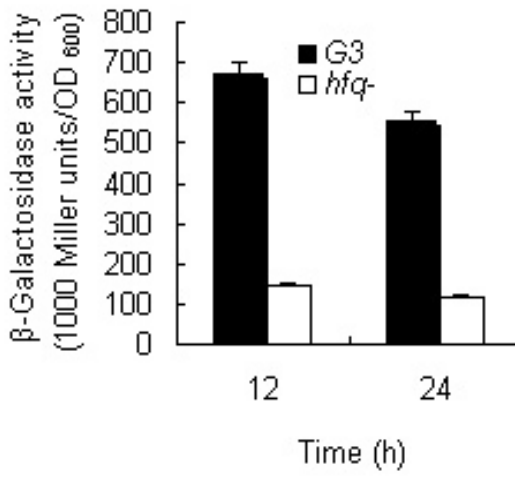

Figure 9. Autocontrol of the *hfq* gene expression by G3. The experiments based on comparative analysis of beta-galactosidase activities when expression of an *hfq'-'lacZ* translational fusion under the background of the wild type G3 and the *hfq*- mutant, respectively.

described to serve as positive regulators of IAA biosynthesis in bacteria (50). Negative control of IAA biosynthesis by QS signaling in strain G3 was described recently (31). Here we provided the first evidence that Hfq is also involved in modulation of IAA biosynthesis in G3. For further exploration of the molecular basis on the Hfq control of IAA production, we have cloned two copies of *ipdC* genes encoding indole-3-pyruvate decarboxylase (IPDC), the key enzyme for IAA biosynthesis in the indole-3-pyruvate (IPyA) pathway from strain G3 to investigate the genetic regulatory networks for IAA biosynthesis.

Pyrrolnitrin (PRN) [3-chloro-4-(2′-nitro-3′-chlorophenyl)-pyrrole] is a tryptophan-derived secondary metabolite with inhibitory effect on the electron transport system of fungi and has been reported to suppress a wide range of fungal and bacterial pathogens (34). Because PRN production is an important biocontrol mechanism against several plant pathogens, extensive work has been carried out to elucidate its gene expression and regulation in *S. plymuthica* and *Pseudomonas fluorescens* Pf-5. Similar to exoenzymes, it has been demonstrated that the involvement of the RpoS and GrrS/GrrA in PRN regulation in IC1270, their mutants displayed markedly less capable of suppressing *Rhizoctonia solani* and *Pythium aphanidermatum* (14). Moreover, the PRN biosynthesis was dependent on AHL signaling in *S. plymuthica* HRO-C48 and *Burkholderia cepacia* complex, characterized with a *lux*-box in promoter region of *prnABCD* operon (15, 51). The RNA-binding protein Hfq has been studied extensively for its function as a modulator of gene expression at the post-transcriptional level. Combining TLC detection of PRN extracts and assay of *lux*- or *lacZ*-based reporter fusions, we provided the evidence that Hfq as a pleiotropic regulator is also involved in control of PRN production. In transcriptional fusions, the reporter gene lacks a promoter, but possesses a functional ribosome binding site, so the reporter gene product is made whenever the "target gene" is being transcribed. This allows us to monitor transcriptional regulation of the target gene and ignores any possible posttranscriptional effects. In translational fusions, the reporter gene lacks both a promoter and a functional ribosome binding site, so the reporter gene product is made whenever the "target gene" is being transcribed and translated (http://lecturer.ukdw.ac.id/dhira/BactGenetics/fusions.html). Consequently, a *prnA* promoter::*lux* fusion showed that the *hfq*- mutant only significantly decreased the level of *prnA* transcription; however, the *hfq*- mutant almost abolished beta-galactosidase activity using an *hfq'-'lacZ* translational fusion and PRN production by TLC detection implied that the control of PRN biosynthesis is at the translational level as well.

In enteric bacteria, Hfq has been shown to regulate the alternative sigma factors such as RpoS, RpoE and RpoH. Consequently, Hfq functions partially via control of these alternative sigma factors. Some of changes in the gene expression pattern are related to reduced translation efficiency of these sigma factors' mRNAs (6, 41). Using a *lacZ'-'rpoS* translational fusion, we also showed that the Hfq deficient mutant in G3 significantly reduced the expressional level of RpoS indicating that Hfq might be involved in control of the transcription of *prnA* indirectly via the stress sigma factor RpoS which has known to regulate PRN production positively in closely related *S. plymuthica* IC1270 (14). Recent findings revealed that sequence elements (ARN) x and A_6 in upstream regions of mRNAs of *rpoS* and *fhlA* are important for Hfq binding and gene regulation *in vivo* (37-39). We also found that the conserved $(ARN)_4$ elements from -188-nt to -177-nt and A_6 from -163 to -158 in the upstream leader region of *rpoS* from G3, suggesting that the mechanisms involved in Hfq-dependent regulation of RpoS might be conserved across different gamma-proteobacteria (Figure 8). However, whether or not the *prn* mRNA is also the direct target of Hfq is still to be verified.

Hfq also has been found to autoregulate its own expression at the translational level by binding two sites on the *hfq* message thereby inhibiting the formation of the translational initiation complex in *E. coli* (5). Both Hfq binding sites A and B followed by a stem-loop structure in the 5'-untranslated region (UTR) of the *hfq* mRNA are contribute to translation auto-repression (18) However our findings demonstrated that Hfq is positive auto-controlled through comparative assays of the *beta*-galactosidase activity of a *hfq'-'lacZ* translational fusion including the entire 5'-UTR up to +57 of *hfq* in the genetic background of the wild type and the *hfq*-.mutant. In *silico* analysis of the 5'-UTR of *hfq* by G3 suggested that after the U-rich stretch UUUUUU in site A, not immediately followed by a stem-loop structures as observed in *E. coli*. However the detailed mechanisms resulting in the differently regulatory patterns remain to be clear.

In bacteria, it is well known that the majority of sRNAs basepair with target mRNAs to regulate their translation and/or decay commonly require the bacterial

Sm-like protein, Hfq. In addition, CsrB family of sRNAs in bacteria have also been studied extensively. They contain multiple CsrA/RsmA binding sites and function as CsrA antagonists by sequestering this homodimeric RNA binding protein to control gene expression post-transcriptionally (4, 40). For example, in *Pseudomonas fluorescent* CHA0, the three CsrB sRNAs homologues, RsmX/Y/Z are involved in global post-transcriptional regulation of biocontrol, including production of antibiotics DAPG and hydrogen cyanide, exoprotease, protection of cucumber from *Pythium ultimum*, and positively regulated swarming motility (52). Recently, it has been shown that CsrA inhibits translation initiation of *hfq* in *E. coli* by binding to a single site overlapping the Shine-Dalgarno sequence (53). Additionally in *P. aeruginosa* the stability and abundance of the small non-coding RNA RsmY is dependent on Hfq (54). Small noncoding RNAs and Hfq also have crucial roles in the regulation of bacterial QS circuits in *Vibrio*, *Pseudomonas* and *Erwinia* (55). However, the *hfq* defective mutant in strain G3 seems no significant impact on production of AHL signals; conversely mutation in a homologue of CsrB-like small RNA caused decreased level of AHL accumulation (data not shown). This variety of interactions place Hfq at a crucial node in bacterial regulatory networks underlying a wide range of cellular processes and pathways (11). Therefore, how the Hfq from the endophytic *S. plymuthica* G3 integrated into the complex regulatory networks to modulate the bacterial physiology and the beneficial interactions with host plants is a future challenge. Additionally, these findings that Hfq involved in differential control of QS circuits, biofilm formation and autotranslation by Hfq in different bacterial species as described above implied that the *hfq*-dependent regulation are genus-specific or species-dependent. The conservative Hfq targets are regulated in opposite directions has also previously been observed in bacteria such as *E. coli*, *S. Typhimurium* and *S. meliloti* (3, 6, 42). These differences may be due to the differences in ecological roles and niches that different bacteria adapt to (42).

All together, our works demonstrate that Hfq as a global regulator plays an important role in the plant beneficial endophyte *S. plymuthica* G3, especially its impact on the production of PRN and IAA, as key determinants for biocontrol and plant growth promotion. Although the underlying molecular mechanisms remain to be investigated, as well as the cross-talk between Hfq and other elements in the complex regulatory networks in *S. plymuthica*, which will help to open a new way to improve the beneficial plant-microbe interactions for plant health promotion via manipulating the post-transcriptional global regulator of biocontrol agents (BCAs) to reduce the use of chemicals in agriculture for ecological conservation and human health.

8. ACKNOWLEDGEMENTS

M. Zhou and K. Gao equally contributed to this article. We are really grateful to Prof. M. Camara, Drs. S. Atkinson and S. Heeb at the University of Nottingham for kindly providing the pDM4, pBluelux and pME6015 plasmids. This work was funded by the National Natural Science Foundation of China (NSFC) (no. 30670030), the NSFC-RS (Royal Society of UK) joint project (no. 30811130218); and the funds from Agricultural Ministry of China (no. 201003004) and the scientific research project from Shandong Province (no. 2010GNC10911).

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Key Words: *Serratia plymuthica*, Hfq, RNA-binding protein, post-transcriptional regulation, biocontrol traits, plant growth promotion, IAA, pyrrolnitrin, autoregulation

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